



Platelet ITAM signaling is critical for vascular integrity in inflammation

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Platelets play a critical role in maintaining vascular integrity during inflammation, but little is known about the underlying molecular mechanisms. Here we report that platelet immunoreceptor tyrosine activation motif (ITAM) signaling, but not GPCR signaling, is critical for the prevention of inflammation-induced hemorrhage. To generate mice with partial or complete defects in these signaling pathways, we developed a protocol for adoptive transfer of genetically and/or chemically inhibited platelets into thrombocytopenic (TP) mice. Unexpectedly, platelets with impaired GPCR signaling, a crucial component of platelet plug formation and hemostasis, were indistinguishable from WT platelets in their ability to prevent hemorrhage at sites of inflammation. In contrast, inhibition of GPVI or genetic deletion of *Clec2*, the only ITAM receptors expressed on mouse platelets, significantly reduced the ability of platelets to prevent inflammation-induced hemorrhage. Moreover, transfusion of platelets without ITAM receptor function or platelets lacking the adapter protein SLP-76 into TP mice had no significant effect on vascular integrity during inflammation. These results indicate that the control of vascular integrity is a major function of immune-type receptors in platelets, highlighting a potential clinical complication of novel antithrombotic agents directed toward the ITAM signaling pathway.

Introduction

Platelets play an essential role in the prevention of excessive blood loss after injury (hemostasis), but can also form occlusive thrombi at sites of atherosclerotic plaque rupture (thrombosis) (1). Platelet plug formation strongly depends on the soluble agonists thrombin, ADP, and thromboxane A₂ (TxA₂), which induce cellular activation via GPCRs (1). Thrombin, generated in response to the exposure of blood to tissue factor in the damaged vascular wall, is crucial in the initiation of thrombus formation. ADP and TxA₂ are released from activated platelets and provide an important positive feedback pathway for sustained platelet activation and platelet recruitment to the growing thrombus. The importance for thrombosis and hemostasis of the various platelet GPCR receptors and their agonists is documented by the protection from thrombotic complications and the increased bleeding tendency observed in patients treated with heparin (which inhibits thrombin), aspirin (which blocks TxA₂ generation), and/or clopidogrel bisulfate (which inhibits one of the ADP receptors, P2Y₁₂) (2). Consistently, mice lacking the major platelet receptors for thrombin and/or ADP exhibit strong protection from experimental thrombosis and markedly prolonged tail bleeding times (3).

The platelet receptor for collagen and laminin, glycoprotein VI (GPVI), is also critical for the initiation of thrombus formation at sites of vascular injury. GPVI belongs to the Ig superfamily of surface receptors and depends on its association with the immunoreceptor

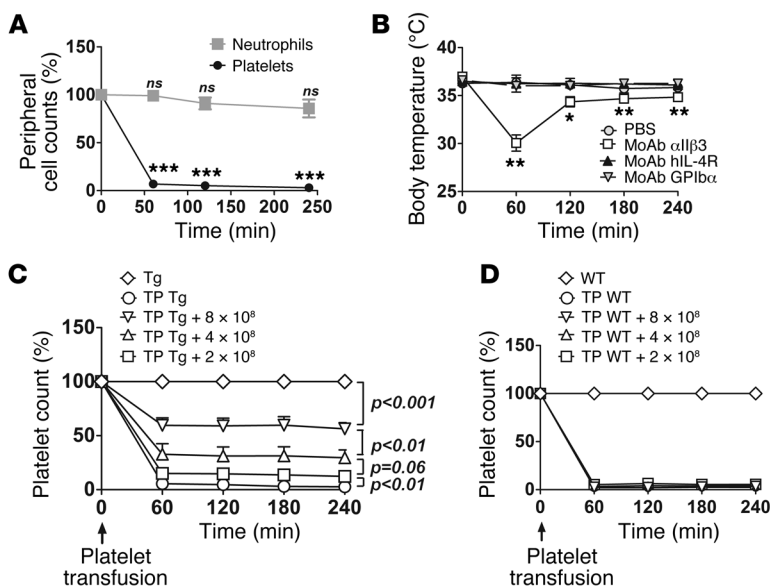
tyrosine activation motif-containing (ITAM-containing) FcR γ chain for signaling (4). Mice deficient in GPVI exhibit a mild defect in hemostasis and moderate to strong protection from thrombosis (5). Patients with impaired GPVI expression/function also show a mild bleeding diathesis (6, 7). Inhibitors of GPVI are currently being evaluated as novel antiplatelet drugs (2). Platelets also express the ITAM receptor C-type lectin-2 (CLEC2), but its role in hemostasis and thrombosis is not well understood. Whereas some investigators demonstrated impaired thrombus formation in the absence of functional CLEC2 (8, 9), others could not reproduce these findings (10). Platelet CLEC2, however, plays a critical role during development. Mice lacking CLEC2 (11, 12) or its ligand, podoplanin (13, 14), have defective separation of their lymphatic and vascular endothelial cells that causes petechia in utero. Similar defects were observed in mice deficient in Syk, Src-homology leucocyte protein 76 (SLP-76), and PLC- γ 2, which are components of the CLEC2 signaling pathway (4).

More recent studies suggested that platelets are also critical for other physiological and pathological processes, such as angiogenesis (15), development (16, 17), immunity (18), viral hepatitis (19), arthritis (20), atherosclerosis (21), and sepsis (22). At sites of inflammation, platelets support leukocyte extravasation (23), but, importantly, they also prevent local hemorrhage. Consequently, thrombocytopenia (TP) is associated with inflammation-induced hemorrhage (24–26). Interestingly, the ability of platelets to safeguard vascular integrity at sites of inflammation is independent of their ability to form a platelet plug, as inflammation-induced hemorrhage was not observed in mice deficient in the major surface adhesion receptors GPIIb-V-IX, integrin $\alpha_{IIb}\beta_3$, or P-selectin (26, 27). Additional mechanistic studies suggest (a) that the platelet support of vascular integrity in inflammation depends on intracellular storage granules and release of the vasoactive compounds stored therein (28), and (b) that platelets protect vascular

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**Figure 1**

Adoptive transfer of WT platelets into TP hIL-4Rα/GPIbα-Tg mice. (A) TP was induced in hIL-4Rα/GPIbα-Tg mice by injection of antibodies against hIL-4R. Peripheral platelet and neutrophil counts were determined at the indicated time points after injection of anti-hIL-4R antibodies ($n = 5$). Results are expressed as percent of count before antibody injection. *** $P < 0.001$ versus 0 minutes. (B) Body temperature was measured over time after injection of anti-hIL-4R or anti-αIIbβ₃ monoclonal antibodies (MoAb) into hIL-4Rα/GPIbα-Tg mice or injection of anti-GPIbα monoclonal antibodies into WT controls ($n = 5$ per antibody). ** $P < 0.01$, * $P < 0.05$ versus PBS. (C and D) Peripheral platelet count in TP hIL-4Rα/GPIbα-Tg (TP Tg; C) or TP WT (D) mice transfused with the indicated amounts of WT platelets ($n = 4$). Results are expressed as percent of count before antibody injection ("Tg" and "WT" groups).

integrity by dampening the inflammatory response (29, 30). However, no information is available on the agonist receptors or the intracellular signaling pathways required for the vessel-stabilizing activity of platelets at sites of inflammation.

TP mice have been a very powerful tool for the identification of novel platelet functions in health and disease (see above). The method most commonly used to render mice TP is based on our previous studies on the cytotoxic effects of antibodies directed toward major platelet surface receptors (31), which demonstrated that antibodies against the glycoprotein Ibα (GPIbα) subunit of the GPIb-V-IX receptor complex and the major integrin receptor on platelets, αIIbβ₃, cause virtually complete TP in mice (31). However, while mice receiving anti-GPIbα antibodies do not show any signs of distress, mice receiving anti-αIIbβ₃ antibodies develop an anaphylaxis-like reaction, with symptoms that include hypothermia and a decrease in hematocrit due to internal bleeding. Thus, antibodies against GPIbα have emerged as the most reliable method to safely render mice TP for studies on the role of platelets in disease. The disadvantage of this method, however, is that the antibody remains in circulation and also targets platelets that are transfused into the TP mice. Consequently, comprehensive studies on platelet signaling molecules that are important for vascular integrity in inflammation would require deletion of multiple genes specifically in the megakaryocyte/platelet lineage using the loxP/PF4-Cre system (32). The technical difficulties, the costs, and the time associated with generation of these mice call for an alternative, more efficient method to generate mice with defects in multiple platelet signaling molecules.

In this study, we used a novel method to generate mice with platelet-specific signaling defects, based on adoptive transfer of platelets into TP mice, to study the platelet signaling responses required for the maintenance of vascular integrity in inflammation.

Results

Adoptive transfer of WT platelets into TP mice. Studying the role of platelet GPCR and ITAM signaling for vascular integrity in inflammation requires a method that allows for the rapid and efficient generation of mice with platelet-specific defects in multiple sig-

naling molecules. Thus, we first established a protocol for the adoptive transfer of genetically modified and/or inhibitor-treated platelets into TP mice. We hypothesized that Tg mice expressing a chimeric human IL-4 receptor α/GPIbα (hIL-4Rα/GPIbα) protein instead of GPIbα on the platelet surface (33) could be rendered TP without side effects by infusion of antibodies against hIL-4Rα. Injection of anti-hIL-4Rα antibody led to virtually complete TP, but not neutropenia, in the hIL-4Rα/GPIbα-Tg mice (Figure 1A). As was the case using antibodies against GPIbα in WT mice (31), anti-IL-4R-induced TP in hIL-4Rα/GPIbα-Tg mice was not associated with hypothermia (Figure 1B). In contrast, infusion of an antibody against αIIbβ₃ into hIL-4Rα/GPIbα-Tg mice induced a significant drop in platelet count and body temperature within 30 minutes (Figure 1B). We next tested whether WT platelets could be successfully transfused into TP hIL-4Rα/GPIbα-Tg mice. Transfusion of 2×10^8 , 4×10^8 , and 8×10^8 WT platelets into TP hIL-4Rα/GPIbα-Tg mice raised the peripheral platelet count to $18.00 \pm 5.76\%$, $29.50 \pm 7.32\%$, and $56.25 \pm 5.81\%$, respectively, of the level in untreated control hIL-4Rα/GPIbα-Tg mice (Figure 1C). In contrast, WT platelets infused into WT mice that received an anti-GPIbα antibody to induce TP were immediately cleared from the circulation (Figure 1D). In summary, we demonstrated that an anti-hIL-4R antibody can be used to safely deplete circulating platelets in hIL-4Rα/GPIbα-Tg mice, facilitating the adoptive transfer of WT platelets into TP mice.

To test the ability of transfused WT platelets to form thrombi at sites of vascular injury, we next studied platelet adhesion to endothelium damaged by laser injury (34). In this model, a thrombus consisting of platelets and fibrin formed rapidly at the site of vascular injury in control WT mice (Figure 2A). We detected fibrin, but not platelets, in injured venules of TP hIL-4Rα/GPIbα-Tg mice (Figure 2A). WT platelets transfused into TP hIL-4Rα/GPIbα-Tg mice were indistinguishable from endogenous platelets in WT control mice in their ability to adhere to and aggregate at sites of vascular injury (Figure 2A). Importantly, transfused platelets also prevented a loss of vascular integrity in models of immune complex-mediated inflammation in the skin (reverse passive Arthus [rPA] reaction) and LPS-induced inflammation in the

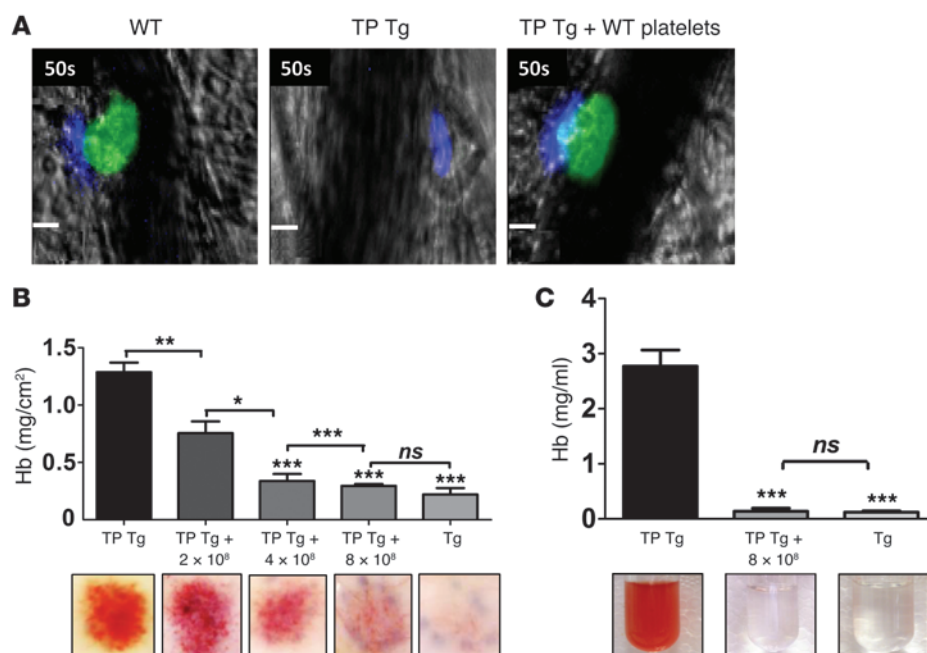


Figure 2

In vivo function of WT platelets transfused into TP hIL-4Rα/GPIbα-Tg mice. **(A)** Representative images of cremasteric venules taken 50 seconds after laser injury in WT mice, TP hIL-4Rα/GPIbα-Tg mice, and TP hIL-4Rα/GPIbα-Tg mice transfused with 8×10^8 WT platelets. Mice were injected with Alexa Fluor 488-labeled antibodies against GPIX to label circulating platelets (green) and Alexa Fluor 647-labeled antibodies against fibrin (blue). Note the complete lack of platelet accumulation in TP hIL-4Rα/GPIbα-Tg mice. Fibrin generation in the same mice demonstrated successful vascular injury. Scale bars: 10 μ m. **(B)** Hb levels in skin lesions 4 hours after rPA challenge in hIL-4Rα/GPIbα-Tg mice and in TP hIL-4Rα/GPIbα-Tg mice reconstituted or not with the indicated amounts of WT platelets ($n = 12$ spots per concentration). Representative lesion sites are also shown. **(C)** Hb levels in BAL 6 hours after LPS challenge in hIL-4Rα/GPIbα-Tg mice and in TP hIL-4Rα/GPIbα-Tg mice reconstituted or not with 8×10^8 WT platelets ($n = 6$ per group). Representative images of BAL are also shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus TP Tg with no platelet reconstitution, or as indicated by brackets.

lung (Figure 2, B and C). In the rPA reaction, mice were challenged by intravenous injection of BSA and intradermal application of anti-BSA antibodies, and bleeding at the site of inflammation was assessed by quantification of tissue hemoglobin (Hb). Compared with unchallenged (0.10 ± 0.008 mg/cm²; data not shown) or rPA-challenged (0.25 ± 0.05 mg/cm²) hIL-4Rα/GPIbα-Tg control mice, tissue Hb content was significantly increased in TP hIL-4Rα/GPIbα-Tg mice 4 hours after rPA challenge (1.28 ± 0.08 mg/cm²; Figure 2B). Transfusion of 2×10^8 , 4×10^8 , and 8×10^8 WT platelets into TP hIL-4Rα/GPIbα-Tg mice dose-dependently decreased tissue Hb at sites of inflammation (0.76 ± 0.10 , 0.47 ± 0.04 , and 0.29 ± 0.03 mg/cm², respectively). To induce inflammation in the lung, mice were challenged by intranasal administration of 7 μ g LPS, and Hb levels were determined in the lavage fluid 6 hours later. Compared with control hIL-4Rα/GPIbα-Tg mice (0.12 ± 0.02 mg/ml), significantly increased Hb levels were observed in the lavage fluid of LPS-challenged TP hIL-4Rα/GPIbα-Tg mice (2.77 ± 0.30 mg/ml; Figure 2C). Consistent with our findings in the rPA model, transfusion of 8×10^8 WT platelets into TP hIL-4Rα/GPIbα-Tg mice before LPS challenge completely prevented hemorrhage in lungs (0.14 ± 0.05 mg/ml; Figure 2C).

Platelet GPCR signaling and vascular integrity in inflammation. To better understand how platelets secure vascular integrity at sites of inflammation, we investigated the roles of platelet GPCRs and ITAM receptors in this process. We first studied platelet preparations with defects in GPCR signaling: (a) platelets isolated from

mice deficient in the thrombin receptor *Par4* (35), (b) platelets isolated from mice treated with clopidogrel to irreversibly inhibit P2Y₁₂, the main receptor for ADP, (c) platelets treated with aspirin to irreversibly inhibit cyclooxygenase-dependent TxA₂ formation, and (d) *Par4*-deficient platelets treated with both clopidogrel and aspirin (referred to herein as *Par4*^{-/-}/clopidogrel/aspirin platelets). To confirm the lack of GPCR response in these platelets, we tested their ability for integrin inside-out activation and α granule release in vitro. As shown in Figure 3A, *Par4*^{-/-}/clopidogrel/aspirin platelets were unresponsive to stimulation with PAR4 receptor-activating peptide (PAR4p) or the combination of the second-wave mediators ADP and TxA₂ (U46619). Both integrin activation and α granule release in *Par4*^{-/-}/clopidogrel/aspirin platelets were partially reduced in response to activation with the snake toxin convulxin and with podoplanin (Figure 3A), which confirmed that full platelet activation via the ITAM receptors GPVI and CLEC2, respectively, requires feedback activation by ADP and TxA₂ (4). Consistent with these in vitro studies, platelets with impaired GPCR signaling were markedly impaired in their ability to form 3-dimensional thrombi at sites of vascular injury (Figure 3B).

Next, all platelet preparations with defects in GPCR signaling were tested for their ability to prevent hemorrhage at sites of rPA-induced inflammation (Figure 4, A and B). 8×10^8 platelets isolated from WT mice, *Par4*^{-/-} mice, clopidogrel-treated WT mice, or clopidogrel-treated *Par4*^{-/-} mice were incubated in the presence or absence of aspirin, washed, and transfused into TP hIL-4Rα/

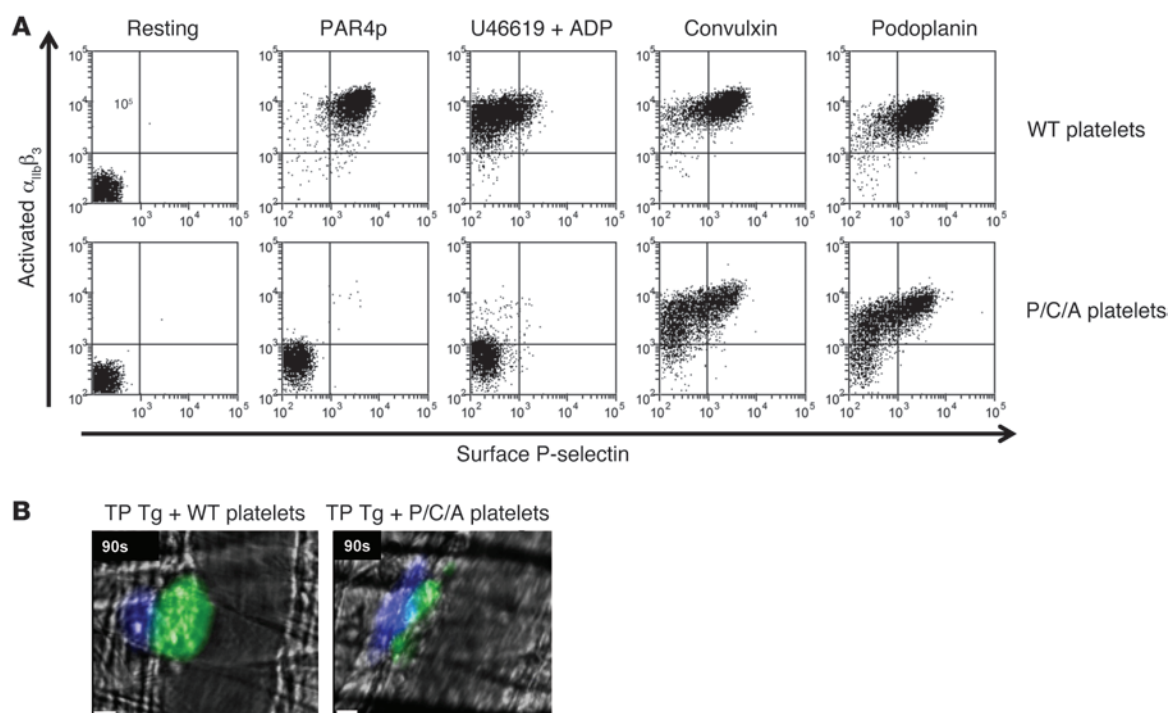


Figure 3

Characterization of platelets with impaired GPCR signaling. (A) WT or *Par4*^{-/-}/clopidogrel/aspirin (P/C/A) platelets were activated with the indicated agonists, stained for activated $\alpha_{IIb}\beta_3$ and surface P-selectin, and analyzed directly by flow cytometry. Data are representative of 3 independent experiments. (B) Representative images of cremasteric venules taken 90 seconds after laser injury in TP hIL-4R α /GPIb α -Tg mice reconstituted with 8×10^8 WT or *Par4*^{-/-}/clopidogrel/aspirin platelets. Mice were injected with Alexa Fluor 488-labeled antibodies against GPIX to label circulating platelets (green) and Alexa Fluor 647-labeled antibodies against fibrin (blue). Scale bars: 10 μ m.

GPIb α -Tg mice before rpA challenge. All platelet transfusions significantly increased the peripheral platelet counts in TP hIL-4R α /GPIb α -Tg mice to approximately 50% of that in control mice (Supplemental Figure 1, A and C). Surprisingly, clopidogrel-inhibited WT platelets, aspirin-inhibited WT platelets, *Par4*^{-/-} platelets, and *Par4*^{-/-}/clopidogrel/aspirin platelets all reduced hemorrhage at sites of rpA-induced inflammation — as assessed by tissue Hb levels (0.26 ± 0.03 , 0.20 ± 0.02 , 0.27 ± 0.04 , and 0.21 ± 0.03 mg/cm², respectively) and macroscopic evaluation — to a level comparable to that observed in TP hIL-4R α /GPIb α -Tg mice transfused with WT platelets (0.29 ± 0.02 mg/cm²; Figure 4, A and B). These results suggested that GPCR signaling in platelets is not critical for maintaining vascular integrity during inflammation. In support of this conclusion, *Par4*^{-/-}/clopidogrel/aspirin platelets were also indistinguishable from WT platelets in their ability to prevent pulmonary hemorrhage in TP hIL-4R α /GPIb α -Tg mice after intranasal administration of LPS (0.15 ± 0.07 and 0.14 ± 0.05 mg/ml Hb, respectively; Figure 4, C and D).

Platelet ITAM signaling and vascular integrity in inflammation. While the GPCR signaling pathway is important for platelet activation by soluble agonists, signaling via ITAM receptors is required for platelet activation by components of the ECM and extravascular cells. Mouse platelets express 2 ITAM receptors: GPVI, a receptor for collagen and laminin in the ECM, and CLEC2, a receptor for podoplanin expressed on extravascular cells (4). To study the role of platelet ITAM signaling in the maintenance of vascular integrity, we tested 4 different platelet preparations: (a) platelets treated in vitro with JAQ1, a blocking monoclonal anti-

body against GPVI (36), (b) platelets isolated from *Clec2*^{-/-} mice, (c) JAQ1-treated *Clec2*^{-/-} platelets, and (d) platelets isolated from mice lacking *Slp76*, known to be downstream of ITAM signaling receptors (37). In vitro, all platelet preparations with defects in ITAM signaling showed normal integrin activation and α granule release in response to the GPCR agonists PAR4p and ADP plus U46619 (Supplemental Figure 2 and Figure 5A). JAQ1-treated WT and *Clec2*^{-/-} platelets were impaired in their responses to the GPVI agonist convulxin and the CLEC2 agonist podoplanin, respectively (Supplemental Figure 2). As expected, integrin activation and α granule release in response to convulxin or podoplanin were abolished in JAQ1-treated *Clec2*^{-/-} and *Slp76*^{-/-} platelets (Supplemental Figure 2 and Figure 5A). As we observed in platelets with impaired GPCR signaling (Figure 3B), *Slp76*^{-/-} platelets were markedly impaired in their ability to form 3-dimensional thrombi in cremasteric venules after laser injury (Figure 5B).

Upon transfusion into TP hIL-4R α /GPIb α -Tg mice, JAQ1-treated WT platelets only partially restored vascular integrity compared with untreated WT platelets at sites of inflammation induced by rpA (0.60 ± 0.10 versus 0.29 ± 0.03 mg/cm² Hb; Figure 6, A and B) or by LPS (1.07 ± 0.10 versus 0.14 ± 0.05 mg/ml Hb; Figure 6, C and D). Platelets isolated from *Clec2*^{-/-} mice also partially reduced hemorrhage in TP hIL-4R α /GPIb α -Tg mice induced by rpA (0.63 ± 0.72 mg/cm² Hb) and LPS (1.62 ± 0.20 mg/ml Hb). Transfusion of JAQ1-treated *Clec2*^{-/-} platelets into TP hIL-4R α /GPIb α -Tg mice had no significant effect on hemorrhage induced by rpA (1.06 ± 0.13 mg/cm² Hb) and LPS (2.42 ± 0.27 mg/ml Hb), and neither did transfusion of *Slp76*^{-/-} platelets

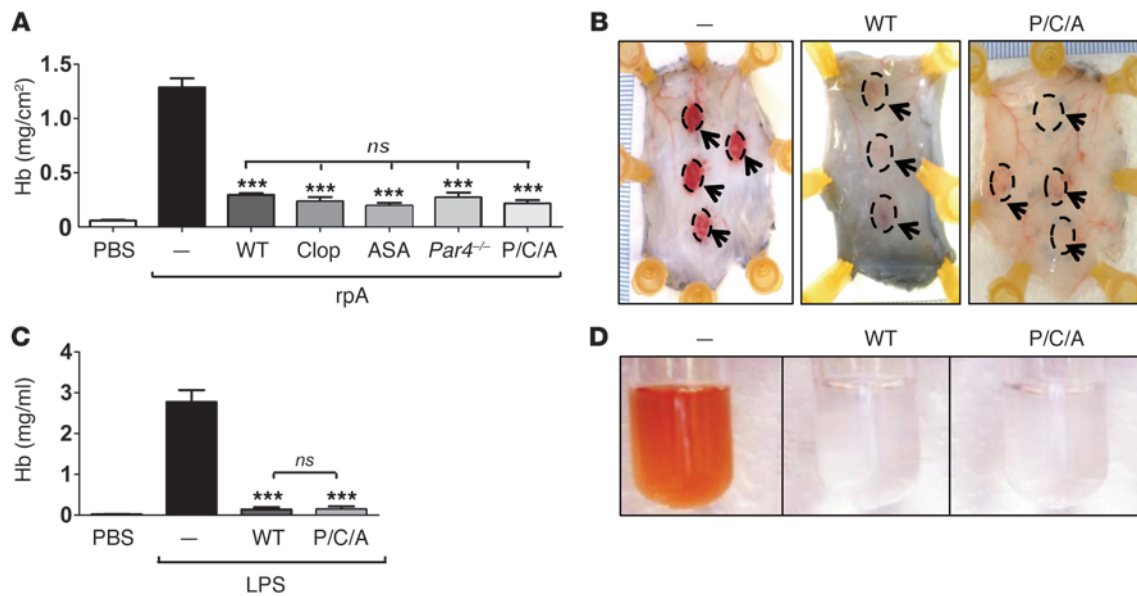


Figure 4

Platelet GPCR signaling is not required for the maintenance of vascular integrity in inflammation. **(A and B)** rpA reaction in skin of TP hIL-4R α /GP1b α -Tg mice reconstituted or not (–) with the indicated platelet preparations (8×10^8 platelets/mouse; $n = 15$ –20 spots per group). Clop, clopidogrel; ASA, aspirin. **(A)** Hb levels in skin lesions 4 hours after rpA challenge. Intradermal injection of PBS (without rpA) is also shown. **(B)** Representative images of rpA reaction sites (indicated by dashed outlines and arrows). **(C and D)** LPS-induced inflammation in lungs of TP hIL-4R α /GP1b α -Tg mice reconstituted or not with the indicated platelet preparations (8×10^8 platelets/mouse; $n = 5$ per group). **(C)** Hb levels in BAL 6 hours after LPS challenge. Intradermal injection of PBS (without LPS) is also shown. **(D)** Representative images of BAL. *** $P < 0.001$ versus no platelet reconstitution.

(Figure 6, A–D). Peripheral platelet counts were similar in mice transfused with the respective platelet preparations (Supplemental Figure 1, B and D), excluding the possibility that hemorrhage at sites of inflammation was the result of impaired survival of platelets after adoptive transfer. These results demonstrated that the protective effect of platelets in the maintenance of vascular integrity during inflammation depends on the 2 ITAM receptors GPVI and CLEC2 and the adapter protein SLP-76.

Discussion

Our studies using a novel method to generate mice with multiple platelet-specific signaling defects identified platelet ITAM signaling as a critical event in the maintenance of vascular integrity at sites of inflammation. Platelets defective in ITAM signaling were unable to prevent hemorrhage at sites of immune complex-induced inflammation in the skin as well as LPS-induced inflammation in the lung. In contrast, platelets defective in GPCR signaling were indistinguishable from WT platelets in their ability to support vascular integrity in both models. Our findings provided evidence that the platelet signaling response required to prevent hemorrhage in inflammation can be generalized for different triggers and vascular beds, although this conclusion will need to be confirmed in other models of inflammation.

The central finding of our work was that the platelet ITAM signaling pathway is critical for the maintenance of vascular integrity in inflammation. Mouse platelets express 2 ITAM receptors, GPVI and CLEC2. Inhibition of GPVI or deficiency in CLEC2 partially reduced the ability of platelets to maintain vascular integrity in immune complex-induced inflammation in the skin or LPS-induced inflammation in the lung. A defect in both GPVI and

CLEC2 signaling, or genetic deletion of the downstream adapter protein SLP-76, completely abolished the positive effect of platelets on vascular integrity. GPVI signaling is likely activated at sites of inflammation by collagen and/or laminin, the 2 physiological ligands for GPVI found in the vessel wall (38). More difficult to explain, however, is how CLEC2 on platelets is activated in these situations. Biochemical and genetic studies have established the transmembrane protein podoplanin as the major CLEC2 ligand. Podoplanin expression was first documented in glomerular podocytes, lymphatic endothelium, and the brain (39). However, podoplanin was not found in blood endothelial cells or pericytes, and smooth muscle podoplanin was identified in advanced, but not early, atherosclerotic lesions (40). Thus, podoplanin is not constitutively expressed in the vessel wall. In LPS-induced inflammation in the lung, platelet CLEC2 may engage podoplanin expressed at high levels in type I epithelial cells in alveolae (41). However, a cellular source for podoplanin has not yet been identified in the skin. Alternatively, podoplanin could be “delivered” to sites of inflammation by infiltrating macrophages, a recently identified source of this molecule (42). Our observations that both GPVI and CLEC2 contributed to a similar extent to platelet-dependent vascular integrity in the lung and skin are consistent with a tissue-independent source of podoplanin as the trigger of platelet CLEC2 signaling. Finally, it is also possible that platelet CLEC2 receptors respond to an as yet unidentified ligand expressed in these tissues or in the vessel wall. Future studies in mice with conditional deletion of podoplanin in various tissues/cell types, such as alveolar cells, lymphatic endothelial cells, monocytes/macrophages, or smooth muscle cells, will be required to distinguish among these possible mechanisms.

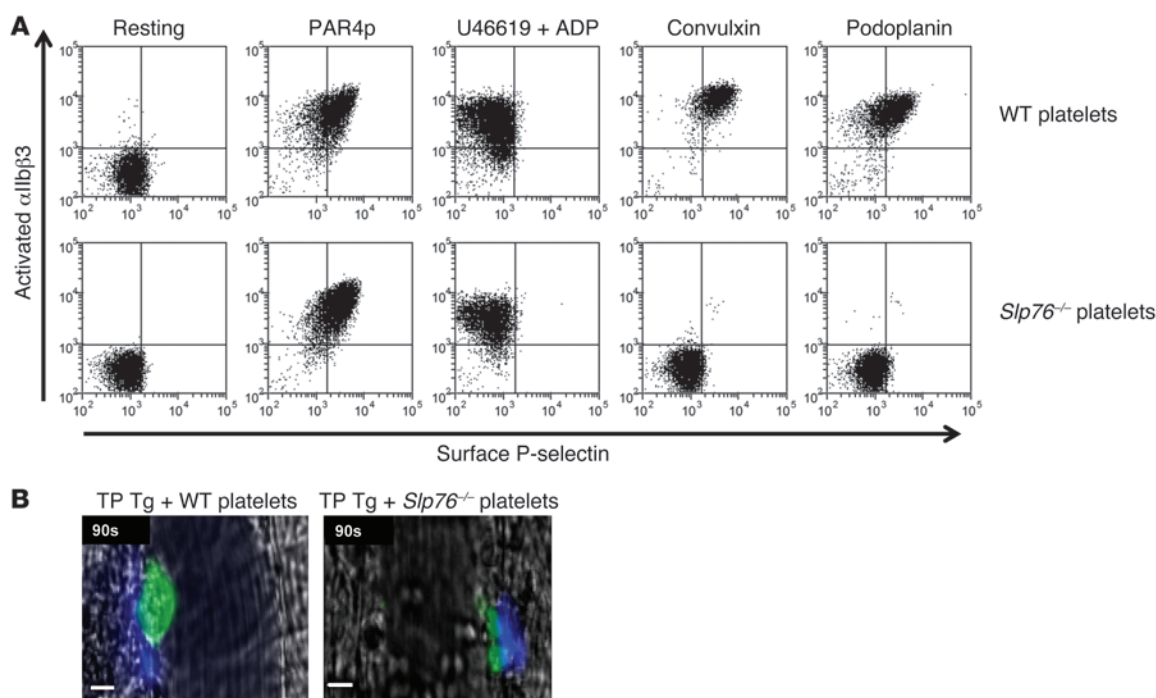


Figure 5

Impaired platelet ITAM signaling and thrombosis in *Slp76*^{-/-} mice. (A) Washed platelets from WT or *Slp76*^{-/-} mice were activated with the indicated agonists, stained for activated $\alpha_{IIb}\beta_3$ and surface P-selectin, and analyzed by flow cytometry. Data are representative of 3 independent experiments. (B) Representative images of cremasteric venules taken 90 seconds after laser injury in TP hIL-4R α /GPIb α -Tg mice reconstituted with 8×10^8 WT or *Slp76*^{-/-} platelets. Mice were injected with Alexa Fluor 488-labeled antibodies against GPIX to label circulating platelets (green) and Alexa Fluor 647-labeled antibodies against fibrin (blue). Scale bars: 10 μ m.

Our results also provided evidence that platelet activation via soluble agonists, such as thrombin, TxA₂, and ADP, is dispensable for the contribution of these cells to the maintenance of vascular integrity. This is surprising, as all 3 agonists play key roles in the ability of platelets to form thrombi at sites of vascular injury (1). Furthermore, ADP and TxA₂ provide critical feedback signaling for platelet activation via ITAM receptors (1). As shown recently, however, platelets contribute to vascular integrity in inflammation and hemostasis (26, 27), but likely depends on the release of vasoactive mediators stored in platelet granules. Activation by ITAM receptors may trigger a specific release reaction different from that induced by GPCRs. Such agonist-specific differences in the released granule content have been described recently, but these studies were focused on the different agonists for GPCRs (43).

Finally, our work highlighted similarities between the molecular mechanisms regulating vascular integrity in inflammation and those facilitating the separation of blood and lymphatic vessels during development. As shown by various groups, lymphatic vascular development during embryogenesis is dependent on platelet activation via the CLEC2/SLP-76 axis (11, 12). Blood-filled lymphatics were also observed in adult mice transplanted with bone marrow from *Clec2*^{-/-} or *Slp76*^{-/-} mice (11). In contrast, no such defects have been described for mice lacking components of the GPCR signaling machinery or for mice deficient in key receptors for platelet adhesion and aggregation. It is important to note, however, that defects in lymphatic vascular development have not been described for mice lacking GPVI, which suggests that the

molecular mechanisms regulating vascular development and vascular integrity in the adult differ, at least with regard to triggering platelet activation.

Critical to our studies was the development of an adoptive transfer system for platelets into TP mice. Here, selective depletion of circulating platelets was achieved in Tg mice with antibodies that recognize hIL-4R, a heterologous antigen expressed on circulating platelets in these mice. With the use of this technique, we were able to rapidly deplete virtually all circulating platelets in hIL-4R α /GPIb α -Tg mice and replace them with platelets defective in all major GPCR or ITAM receptors. Importantly, this antibody-mediated depletion of platelets did not affect neutrophils or other blood cells (data not shown). In comparison, peripheral platelet counts in mice lacking the thrombopoietin receptor c-Mpl, a key regulator of megakaryocytopoiesis, are only reduced by approximately 90% compared with controls (44). The remaining platelets are fully functional, and *c-mpl*^{-/-} mice do not bleed. Furthermore, c-Mpl deficiency also affects other hematopoietic progenitor cells and thus does not represent a platelet-specific model. Mice lacking the transcription factor p47 NF-E2 (45, 46) have near-complete TP due to a defect on thrombopoiesis, which leads to excessive hemorrhage and perinatal lethality. In addition, p47 NF-E2 knockout mice show several red blood cell defects. Finally, TP induced by chemotherapeutic agents, such as 1,4-butanediol dimethanesulfonate (Busulfan; ref. 47), is accompanied by leukopenia, and thus cannot be used for studying inflammation in mice.

In summary, we here described the comprehensive analysis of the platelet signaling response required to maintain vascular integ-

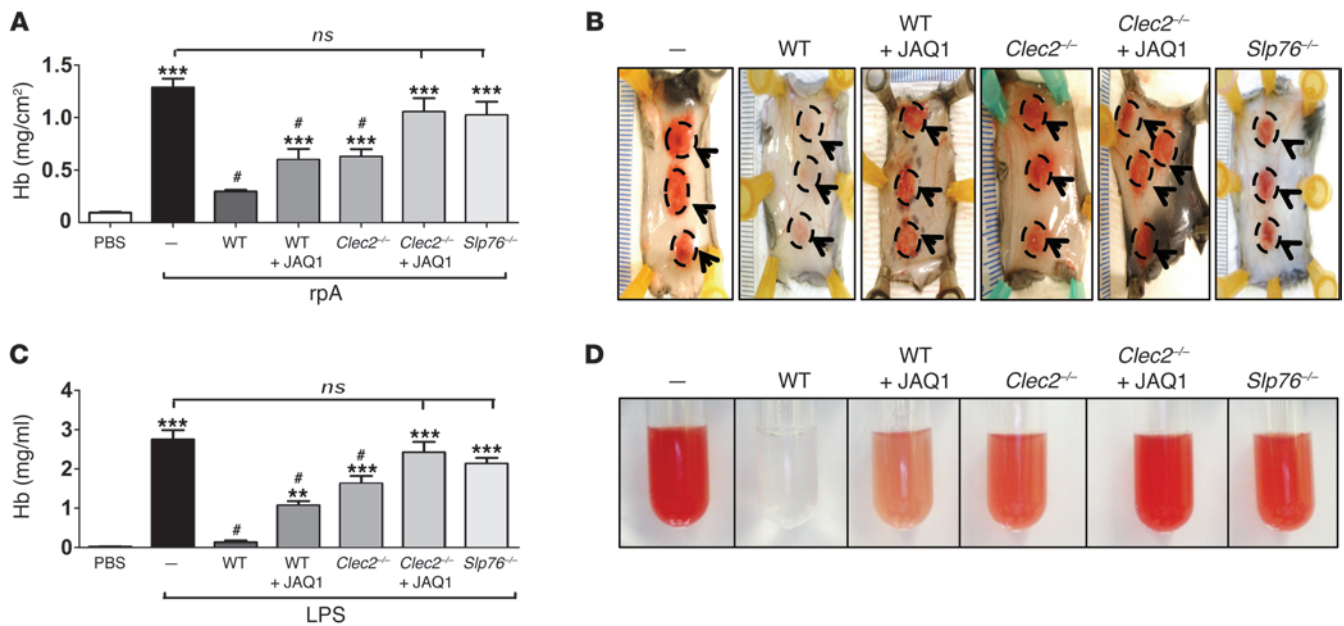


Figure 6

Platelet ITAM signaling is critical for the maintenance of vascular integrity during inflammation. (A and B) Hemorrhage in skin lesions of TP hIL-4R α /GPIb α -Tg mice reconstituted or not with the indicated platelet preparations (8×10^8 platelets/mouse), with or without treatment with JAQ1 antibody ($n = 15$ –20 spots per group). (A) Hb levels 4 hours after rpA challenge. Intradermal injection of PBS (without rpA) is also shown. (B) Representative images of lesions (dashed outlines and arrowheads). (C and D) LPS-induced inflammation in lungs of TP hIL-4R α /GPIb α -Tg mice reconstituted or not with the indicated platelet preparations (8×10^8 platelets/mouse), with or without treatment with JAQ1 antibody ($n = 3$ –5 per group). (C) Hb levels in BAL after LPS challenge. Intradermal injection of PBS (without LPS) is also shown. (D) Representative images of BAL. ** $P < 0.01$, *** $P < 0.001$ versus WT platelets; # $P < 0.001$ versus no platelet reconstitution.

ity in inflammation. By generating mice with multiple platelet-specific signaling defects, we demonstrated that vascular integrity in inflammation depended on both ITAM receptors, GPVI and CLEC2, as well as the adapter protein SLP-76. In contrast, signaling via the major GPCRs was dispensable for this response. Our studies highlight potential complications associated with novel antiplatelet drugs targeting the ITAM signaling pathway, which may lead to blood-lymph mixing or to bleeding at sites of inflammation.

Methods

Animals. 6- to 8-week-old C57BL/6 WT mice were purchased from Jackson laboratory. *Par4*^{-/-} mice (35) and hIL-4R α /GPIb α -Tg mice (33) were bred and housed in our animal facility. Where indicated, WT and *Par4*^{-/-} mice were treated with clopidogrel 24 and 3 hours before the experiment at a dosage of 75 mg/kg body weight (34). *Clec2*^{-/-} and *Slp76*^{fl/fl};PF4-Cre chimeric (*Slp76*^{-/-}) mice (11, 37) were generated by transplant of the respective bone marrow into WT mice lethally irradiated with 2 doses of 6.5 Gy 4 hours apart (13 Gy total) using a Cs¹³⁷ irradiator (J.L. Shepherd).

Reagents and antibodies. Low-molecular weight enoxaparin sodium (Lovenox) and clopidogrel (Plavix) were purchased from Sanofi-Aventis, and heparin-coated capillaries were purchased from VWR. Aspirin was purchased from Bayer. Hb, BSA, prostacyclin (PGI₂), LPS, ADP, red blood cell lysis buffer, and formic acid were purchased from Sigma-Aldrich. The following antibodies were used: anti-GPIb α , GPIX, GPVI, and JON/A-PE (Emfret Analytics); recombinant mouse podoplanin/Fc chimera protein and anti-hIL-4R (clone 25463; R&D Systems); anti- $\alpha_{IIb}\beta_3$, Ly6G, and FITC-conjugated anti-P-selectin (BD Biosciences); anti-BSA (MP Biomedical). Alexa Fluor 647-labeled antibodies against fibrin were a gift from R. Camire (Children's Hospital of Philadelphia, Philadelphia, Pennsylvania,

USA). Convulxin was provided by K.J. Clemetson (Theodor Kocher Institute, Bern, Switzerland), and PAR4p was purchased from Advanced Chemtech. U46619 was purchased from Cayman Chemical.

Platelet depletion. TP was induced in WT or hIL-4R α /GPIb α -Tg mice by retro-orbital injection of antibodies against GPIb α and hIL-4R, respectively (2.5 μ g/g body weight). Peripheral platelet and neutrophil counts were assessed by flow cytometry 4 hours after antibody injection (see below).

Platelet transfusion. Blood was drawn into heparinized tubes from the retro-orbital plexus of sedated mice (7.7 μ l/g body weight). Platelet-rich plasma (PRP) was obtained by centrifugation at 100 g for 5 minutes. PRP was centrifuged at 700 g in the presence of PGI₂ (2 μ g/ml) for 5 minutes at room temperature. After 1 washing step, pelleted platelets were resuspended in modified Tyrode's buffer (137 mM NaCl, 0.3 mM Na₂HPO₄, 2 mM KCl, 12 mM NaHCO₃, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 5 mM glucose, pH 7.3). Platelets from several donor mice were pooled, and the platelet count was adjusted to 1×10^9 , 2×10^9 , or 4×10^9 platelets/ml. BSA was added to purified platelets to yield a final BSA concentration of 150 μ g/g body weight in mice. Recipient mice were injected with 200 μ l washed platelets and BSA.

Flow cytometry. For determination of endogenous and transfused platelet counts, diluted whole blood was stained with antibodies against GPIb α (phycoerythrin labeled; 2 μ g/ml) and GPIX (Alexa Fluor 488 labeled; 2 μ g/ml), and counts were assessed by flow cytometry (Accuri C6; BD Biosciences). To document the selective depletion of hIL-4R α /GPIb α -Tg platelets by anti-hIL-4R antibodies, we transfused WT platelets into hIL-4R α /GPIb α -Tg mice, followed by injection of anti-hIL-4R antibodies (Supplemental Figure 3).

For determination of peripheral neutrophil counts, 50 μ l heparinized whole blood was lysed in red cell lysis buffer for 10 minutes at room temperature. The lysed cells were centrifuged at 250 g, and the supernatant



was carefully discarded. The pellet was resuspended in 20 μ l PBS, and cells were stained with 2 μ g/ml Alexa Fluor 488-labeled anti-Ly6G antibody (10 minutes at room temperature). Cells were analyzed immediately by flow cytometry. Results were expressed relative to neutrophil count before antibody injection.

Platelet activation. Platelets were washed and diluted with Tyrode's buffer containing 1 mM CaCl_2 ; stimulated with PAR4p (300 μ M), ADP (10 μ M) plus U46619 (5 μ M), convulxin (300 ng/ml), or recombinant mouse podoplanin/Fc (1 μ g/ml) plus anti-Fc IgG for 10 minutes; and stained for activated $\alpha_{\text{IIb}}\beta_3$ (JON/A-PE; 2 μ g/ml) and surface expression of P-selectin (anti-P-selectin-FITC; 2 μ g/ml). Where indicated, diluted washed platelets were incubated with JAQ1 (20 μ g/ml) or aspirin (1 mg/ml) for 20 minutes prior to activation. The samples were directly analyzed on an Accuri C6 flow cytometer.

rpA reaction. The rpA reaction was triggered by intradermal injection of anti-BSA antibodies (60 μ g diluted in PBS) followed by retro-orbital injection of Tyrode's buffer or washed platelets resuspended in Tyrode's buffer containing BSA, to reach a BSA concentration of 150 μ g/g body weight. 4 hours later, mice were sacrificed, skin biopsies (8-mm punch) were homogenized in 500 μ l PBS and spun at 15,000 g for 10 minutes, and the supernatant was analyzed. Formic acid was added, and the optical density at 405 nm was measured. Diluted bovine Hb was used to set up a standard curve.

Lung inflammation model. Mice were sedated and inoculated intranasally with 7 μ g *Pseudomonas aeruginosa* LPS or PBS vehicle, as described previously (27). 10 minutes after intranasal application, washed platelets were injected retro-orbitally into mice. BAL was performed 6 hours later by cannulating the trachea with an 18-gauge angiocath, and lungs were lavaged 3 times with 1 ml cold sterile PBS. Lavage fluids were pooled, and Hb concentration was measured as described above.

Inhibitor studies. The collagen receptor GPVI was inhibited by incubation of platelets in vitro with the blocking antibody JAQ1 (100 μ g/ml; 15 minutes). Unbound JAQ1 antibody was washed away prior to platelet transfection. To inhibit cyclooxygenase-mediated generation of TxA_2 , platelets were treated with aspirin (1 mg/ml) for 20 minutes at 37°C, washed, and then injected into recipient mice.

Laser injury-induced thrombosis model. Laser-induced thrombosis in the cremaster muscle microcirculation was performed as described previously (34). Briefly, male mice (12–14 weeks of age) were anesthetized by intraperitoneal injection of Avertin (0.25 mg/g body weight). The cremaster muscle was isolated, and the microvessels were studied with a Zeiss Examiner Z1 microscope. Laser injuries were inflicted with an Ablate 3i system. 5 minutes prior to injury, mice were injected intravenously with Alexa Fluor 488-labeled antibodies against GPIX (0.3 μ g/g body weight) and Alexa Fluor 647-labeled antibodies against fibrin (0.45 μ g/g body weight). Images were collected for 5 minutes at a speed of 1 frame per second using Slidebook software.

Statistics. 2-group comparison of parametric data was compared using a 2-tailed Student's *t* test. Statistical significance among multiple groups was assessed by 1-way ANOVA with Bonferroni post-hoc analysis. Statistical analyses were performed using Prism software (GraphPad). A *P* value of 0.05 or less was considered significant. All data are presented as mean \pm SEM.

Study approval. All animal experiments and protocols were reviewed and approved by, and performed in accordance with, the Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

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